\TENT COOPERATION TRI Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 January 2000 (14.01.00)	
International application No. PCT/FI99/00505	**Applicant's or agent's file reference** SPRV 2 PCT
International filing date (day/month/year) 09 June 1999 (09.06.99)	**Priority date** (day/month/year) 10 June 1998 (10.06.98)
Applicant TÖLÖ, Hannele et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

22 November 1999 (22.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer A. Karkachi Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

3052650

PCT REQUEST

SPRV 2 PCT

Original (for **SUBMISSION**) - printed on 09.06.1999 09:12:20 AM

0	**For receiving Office use only**	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	

0-4	**Form - PCT/RO/101 PCT Request**	
0-4-1	Prepared using	PCT-EASY Version 2.83 (updated 01.03.1999)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	National Board of Patents and Registration (Finland) (RO/FI)
0-7	**Applicant's or agent's file reference**	SPRV 2 PCT
I	**Title of invention**	METHOD FOR PREPARING VIRUS-SAFE PHARMACEUTICAL COMPOSITIONS
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	SUOMEN PUNAINEN RISTI VERIPALVELU
II-5	Address:	Kivihaantie 7 FIN-00310 Helsinki Finland
II-6	State of nationality	FI
II-7	State of residence	FI
III-1	**Applicant and/or inventor**	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	TÖLÖ, Hannele
III-1-5	Address:	Ulvilantie 16 C FIN-00350 Helsinki Finland
III-1-6	State of nationality	FI
III-1-7	State of residence	FI

PCT REQUEST SPRV 2 PCT

Original (for **SUBMISSION**) - printed on 09.06.1999 09:12:20 AM

III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	PARKKINEN, Jaakko
III-2-5	Address:	Liinasaarentie 21 B FIN-02160 Espoo Finland
III-2-6	State of nationality	FI
III-2-7	State of residence	FI
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name	SEPPO LAINE OY
IV-1-2	Address:	Itämerenkatu 3 B FIN-00180 Helsinki Finland
IV-1-3	Telephone No.	+358-9-68 59 560
IV-1-4	Facsimile No.	+358-9-68 595 610
IV-1-5	e-mail	seppo.laine@selpat.fi
V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW SD SZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AL AM AT AU AZ BA BB BG BR BY CA CH&LI CN CU CZ DE DK EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT UA UG US UZ VN YU ZA ZW

PCT REQUEST SPRV 2 PCT

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V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	**Exclusion(s) from precautionary designations**	NONE	
VI-1	**Priority claim of earlier national application**		
VI-1-1	Filing date	10 June 1998 (10.06.1998)	
VI-1-2	Number	981337	
VI-1-3	Country	FI	
VI-2	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1	
VII-1	**International Searching Authority Chosen**	Swedish Patent Office (ISA/SE)	
VIII	**Check list**	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description	14	-
VIII-3	Claims	2	-
VIII-4	Abstract	1	sprv2.txt
VIII-5	Drawings	3	-
VIII-7	TOTAL	24	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-9	Separate signed power of attorney	✓	-
VIII-16	PCT-EASY diskette	-	diskette
VIII-17	Other (specified):	copy of office action	-
VIII-18	**Figure of the drawings which should accompany the abstract**		
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant or agent**		
IX-1-1	Name	SEPPO LAINE OY	
IX-1-2	Name of signatory	Christoffer Sundman	

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10-1	**Date of actual receipt of the purported international application**	

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Original (for **SUBMISSION**) - printed on 09.06.1999 09:12:20 AM

10-2 10-2-1 10-2-2	**Drawings:** Received Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/SE
10-6	**Transmittal of search copy delayed until search fee is paid**	

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11-1	**Date of receipt of the record copy by the International Bureau**	

PATENT COOPERATION TREATY

PCT

REC'D 28 SEP 2000

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference SPRV 2 PCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/FI99/00505	International filing date *(day month year)* 09.06.1999	Priority date *(day month year)* 10.06.1998
International Patent Classification (IPC) or national classification and IPC7 C 07 K 1/34, C 07 K 14/56, A 61 K 38/21		
Applicant SUOMEN PUNAINEN RISTI VERIPALVELU et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22.11.1999	12.09.2000
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Telex 17978 PATOREG-S Facsimile No. 08-667 72 88	Authorized officer Henrik Nilsson/Eö Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00505

I. Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*

☐ the international application as originally filed.

☒ the description, pages 1-14 . as originally filed.
pages _____ . filed with the demand.
pages _____ . filed with the letter of _____ .
pages _____ . filed with the letter of _____ .

☒ the claims. Nos. _____ . as originally filed.
Nos. _____ . as amended under Article 19.
Nos. _____ . filed with the demand.
Nos. 1-15 . filed with the letter of 30.08.2000 .
Nos. _____ . filed with the letter of _____ .

☒ the drawings. ~~sheets~~/fig 1-3 . as originally filed.
~~sheets~~/fig _____ . filed with the demand
~~sheets~~/fig _____ . filed with the letter of _____ .
~~sheets~~/fig _____ . filed with the letter of _____ .

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, ~~sheets~~/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-15	YES
	Claims		NO
Inventive step (IS)	Claims		YES
	Claims	1-15	NO
Industrial applicability (IA)	Claims	1-15	YES
	Claims		NO

2. Citations and explanations

The invention relates to a method of preparing virus-safe pharmaceutical compositions of interferon, and to a method of stabilising such compositions. The invention comprises the steps of adding a non-ionic detergent to the solution, filtering the solution on a virus removal filter with a pore size of 10-40 nm and recovering the filtrate.

The claims have been redrafted after the Written Opinion.

The International Search revealed three documents of particular relevance:

A. EP152345 A2
B. EP231816 A2
C. EP571871 A2

Document A discloses a pharmaceutical composition containing interferon. The composition contains $25 \cdot 10^3$ - $50 \cdot 10^6$ units of interferon per millilitre. The composition further contains 10% of a 3% solution of the non-ionic detergent polysorbate 80 (see example 2), which equals a polysorbate 80 concentration of 0.3 g/l. However, the composition is sterile filtered, and it is not disclosed in document A that the composition is virus-safe.

Document B discloses a pharmaceutical composition containing $1 \cdot 10^8$ units of interferon per millilitre. The composition further contains 1g/l polyoxyethylene sorbitan monolaureate (polysorbat 20). However, the composition is sterile filtered, and it is not disclosed in document B that the composition is virus-safe.

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

Document C discloses membranes that can be used for sterile filtration of solutions. It is disclosed that the membranes may have a pore size as small as 10 nm.

As disclosed in documents A and B the problem of stabilising an interferon composition has been solved using non-ionic detergents, such as polysorbate 80. Furthermore, it is considered well known to the person skilled in the art that virus-free solutions may be obtained by filtration processes employing filters with sufficiently small pore sizes. As indicated in document C, membranes with the pore sizes disclosed in the present application (10 nm) are known to the person skilled in the art and have been used for sterile filtering solutions. Sterile filtration is the method of filtration used for the preparation of interferon solutions disclosed in documents A and B. The effect obtained by using the filters disclosed in document C in the filtration processes disclosed in documents A and B can be expected to be virus removal, due to the small membrane pore size.

Further, it is considered obvious to the person skilled in the art to make interferon solutions comprising any interferon subtypes. Thus, claims 1-15 are not considered to fulfil the requirement of inventive step.

Claims 1-15 are considered to be industrially applicable.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference SPRV 2 PCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
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International Patent Classification (IPC) or national classification and IPC7 C 07 K 1/34, C 07 K 14/56, A 61 K 38/21		
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Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00505

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-15	YES
	Claims		NO
Inventive step (IS)	Claims		YES
	Claims	1-15	NO
Industrial applicability (IA)	Claims	1-15	YES
	Claims		NO

2. Citations and explanations

The invention relates to a method of preparing virus-safe pharmaceutical compositions of interferon, and to a method of stabilising such compositions. The invention comprises the steps of adding a non-ionic detergent to the solution, filtering the solution on a virus removal filter with a pore size of 10-40 nm and recovering the filtrate.

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Document B discloses a pharmaceutical composition containing $1 \cdot 10^8$ units of interferon per millilitre. The composition further contains 1g/l polyoxyethylene sorbitan monolaureate (polysorbat 20). However, the composition is sterile filtered, and it is not disclosed in document B that the composition is virus-safe.

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

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As disclosed in documents A and B the problem of stabilising an interferon composition has been solved using non-ionic detergents, such as polysorbate 80. Furthermore, it is considered well known to the person skilled in the art that virus-free solutions may be obtained by filtration processes employing filters with sufficiently small pore sizes. As indicated in document C, membranes with the pore sizes disclosed in the present application (10 nm) are known to the person skilled in the art and have been used for sterile filtering solutions. Sterile filtration is the method of filtration used for the preparation of interferon solutions disclosed in documents A and B. The effect obtained by using the filters disclosed in document C in the filtration processes disclosed in documents A and B can be expected to be virus removal, due to the small membrane pore size.

Further, it is considered obvious to the person skilled in the art to make interferon solutions comprising any interferon subtypes. Thus, claims 1-15 are not considered to fulfil the requirement of inventive step.

Claims 1-15 are considered to be industrially applicable.

Claims:

1. Method of preparing a virus-safe pharmaceutical composition of a biologically active protein selected from the group of interferons, comprising the steps of

- adding to a solution of the protein a non-ionic detergent in an efficient amount to provide an extended shelf-life of the pharmaceutical composition;
- subjecting the solution containing the non-ionic detergent to filtration on a virus removal filter with a pore size of 10 to 40 nm; and
- recovering the filtrate.

2. The method according to claim 1, wherein the non-ionic detergent is selected from the group consisting of polyoxyethylene sorbitan mono-oleate, polyoxyethylene sorbitan monolaurate and polyoxyethylene lauryl ether.

3. The method according to claim 2, wherein the non-ionic detergent comprises polyoxyethylene sorbitan mono-oleate (polysorbate 80), which is added in an amount exceeding the critical micellar concentration.

4. The method according to claim 3, wherein polysorbate is added in an amount of 0.05 to 1 g/l.

5. The method according to any of claims 1 to 4, wherein the pharmaceutical composition comprises the solution of purified α-interferon.

6. The method according to any of claims 1 to 5, wherein the activity of the α-interferon solution before virus filtration is in the range of 3 to 50 mill. IU/ml.

7. The method according to claim 5 or 6, wherein the pharmaceutical composition comprises an α-interferon solution containing at least one α-interferon subtype selected from the group consisting of α1, α2, α4, α7, α8, α10, α14, α17 and α21.

8. The method according to any of the preceding claims, comprising preparing a pharmaceutical composition comprising purified leukocyte or lymphoblastoid α-interferon essentially in the absence of α-interferon polymers and albumin-interferon complexes.

9. The method according to any of the preceding claims, comprising prefiltering a proteineous solution with a 0.04-0.2 μm filter, then filtering it with a virus removal filter having a pore size of 10-40 nm, and finally subjecting the filtrate to sterile filtration, and recovering the filtrate.

10. The method according to any of claims 1 to 8, comprising sterile filtering a proteineous solution and subsequently subjecting the filtrate of the sterile filtration to virus removal filtration with a filter having a pore size of 10 to 40 nm, and recovering the filtrate.

11. The method according to any of claims 1 to 10, comprising using a virus removal filter capable of reducing the concentration of model viruses having a size of ca 20 to ca 40 nm with at least 4 log during a spiking test.

12. Method of stabilizing pharmaceutical compositions of purified leukocyte α-interferon subjected to filtration on a virus removal filter, comprising using a polysorbate as a stabilizer.

13. A virus-safe α-interferon composition, comprising a non-ionic detergent as a stabilizer in an amount exceeding the critical micellar concentration of the detergent and being essentially free from substances and agents retained on a virus-filter having a high virus retentive capacity even for small non-enveloped viruses.

14. The composition according to claim 13, comprising an α-interferon solution containing at least one α-interferon subtype selected from the group consisting of α1, α2, α4, α7, α8, α10, α14, α17 and α21, and containing a polysorbate as a stabilizer in an amount of 0.05 to 1 g/l.

15. The composition according to claim, comprising an α-interferon solution containing at least two α-interferon subtypes selected from the group consisting of α1, α2, α4, α7, α8, α10, α14, α17 and α21.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00505

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/34, C07K 14/56, A61K 38/21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0152345 A2 (INTERFERON SCIENCES, INC A DELAWARE CORPORATION), 21 August 1985 (21.08.85), see abstract; page 17, line 25 - page 18, line 1; example 2 --	1-16
X	EP 0231816 A2 (DR. KARL THOMAS GMBH), 12 August 1987 (12.08.87), see abstract; example 5 --	1-16
A	EP 0571871 A2 (SEITZ-FILTER-WERKE GMBH UND CO.), 1 December 1993 (01.12.93), see abstract; page 3, lines 10-14; page 6, lines 16-20 -- --------	1-16

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 Sept 1999	15 -09- 1999
Name and mailing address of the ISA: Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/EÖ Telephone No. + 46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/08/99

International application No.
PCT/FI 99/00505

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0152345 A2	21/08/85	SE 0152345 T3	
		AT 91901 T	15/08/93
		CA 1254832 A	30/05/89
		DE 3587479 A,T	02/09/93
		JP 1978803 C	17/10/95
		JP 7005479 B	25/01/95
		JP 60188328 A	25/09/85
		US 4680175 A	14/07/87
		US 4911908 A	27/03/90
EP 0231816 A2	12/08/87	SE 0231816 T3	
		AT 63823 T	15/06/91
		AU 601712 B	20/09/90
		AU 6829287 A	06/08/87
		CA 1295242 A	04/02/92
		DD 284602 A	21/11/90
		DE 3603444 A	06/08/87
		DK 58387 A	06/08/87
		DK 164202 B,C	25/05/92
		FI 86144 B,C	15/04/92
		FI 870457 A	06/08/87
		GR 3002270 T	30/12/92
		IE 59697 B	23/03/94
		JP 62209024 A	14/09/87
		PH 24377 A	13/06/90
		PT 84243 A,B	01/03/87
EP 0571871 A2	01/12/93	DE 4217335 A,C	02/12/93
		DE 59304079 D	00/00/00
		ES 2092725 T	01/12/96
		US 5376274 A	27/12/94

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B 9518 (N,N-bis[3-D-Gluconamidopropyl]-cholamide)
Min. 95%
Useful in solubilization of functional opiate receptor.
Ref.: Hjelmeland, L.M., et al., Anal. Biochem., **130**, 485 (1983).
[86303-22-2] $C_{44}H_{75}N_3O_{18}$ FW 878.1

DECANOYL-N-METHYLGLUCAMIDE — 500 mg 14.40; 1 g 25.20; 5 g 97.80; 25 g 359.70
D 6277 (MEGA-10)
Approx. 98%
[85261-20-7] $C_{17}H_{35}NO_6$ FW 349.5

n-DECYL β-D-GLUCOPYRANOSIDE — 250 mg 18.90; 1 g 48.20; 5 g 188.05
D 5394 **Approx. 98%**
[58846-77-8] $C_{16}H_{32}O_6$ FW 320.4

n-DECYL β-D-MALTOPYRANOSIDE — 500 mg 27.00; 1 g 44.95; 5 g 179.95
D 7658 **Approx. 97%**
[82494-09-5] $C_{22}H_{42}O_{11}$ FW 482.6

DEOXY-BIGCHAP (pfs) — 100 mg 22.00; 500 mg 87.10
D 9414 (N,N-bis[3-Gluconamidepropyl] deoxycholamide)
>65% (TLC)
[105496-19-3] $C_{42}H_{75}N_3O_{15}$ FW 862.1

DIETHYLENE GLYCOL MONOPENTYL ETHER (pfs) — 500 mg 24.40; 1 g 36.40; 5 g 141.25
D 3023 Useful in solubilizing membrane proteins.
Ref.: 1. LeMaire, M. et al., Eur. J. Biochem., **129**, 529 (1983)
2. Hayter, J.B. and Zulauf, M., Coll. Polymer Sci., **260**, 1023 (1982).
[18912-61-7] $C_9H_{20}O_3$ FW 176.3

DIGITONIN
(Digitin)
[11024-24-1] FW 1229.3

Approx. 50% (TLC) (pfs) — 250 mg 14.05; 1 g 35.20; 5 g 136.75; 25 g 645.85; 100 g 2451.00 FOB Sigma
D 5628 Suitable for cholesterol determination.
For digitonin recommended for use in aqueous solution, see D 1407.

Approx. 50% (TLC) (pfs) — 250 mg 14.80; 1 g 44.00; 5 g 172.40 FOB Sigma
D 1407 Recommended for use in aqueous solutions. See insert for details.

n-DODECYL β-D-GLUCOPYRANOSIDE — 100 mg 10.25; 250 mg 20.45; 1 g 56.70; 5 g 208.75
D 8035 (n-Dodecyl glucoside)
Minimum purity: 97%
[59122-55-3] $C_{18}H_{36}O_6$ FW 348.5

n-DODECYL β-D-MALTOSIDE — 500 mg 33.15; 1 g 55.15; 5 g 220.50; 25 g 873.15
D 4641 *[69227-93-6]* $C_{24}H_{46}O_{11}$ FW 510.6

HEPTANOYL-N-METHYLGLUCAMIDE — 500 mg 14.40; 1 g 25.20; 5 g 97.80; 25 g 359.70
H 1639 (MEGA-7)
Approx. 98%
[101397-87-9] $C_{14}H_{29}NO_6$ FW 307.4

n-HEPTYL β-D-GLUCOPYRANOSIDE — 500 mg; 1 g; 5 g
H 1389 **Approx. 98%**
[78617-12-6] $C_{13}H_{26}O_6$ FW 278.3

n-HEPTYL β-D-THIOGLUCOSIDE (pfs) — 100 mg; 250 mg; 1 g
H 3264 *[85618-20-8]* $C_{13}H_{26}O_5S$ FW 294.4

n-HEXYL β-D-GLUCOPYRANOSIDE — 500 mg; 1 g; 5 g
H 9016 **Minimum purity 95%**
[59080-45-4] $C_{12}H_{24}O_6$ FW 264.3

1-MONOOLEOYL-rac-GLYCEROL — 25 g; 50 g; 100 g; 500 g; 1 kg
M 7765 (1-Mono-[(cis)-9-octadecenoyl]-rac-glycerol, Glyceryl cis-9-octadecanoate; Monoolein)
Approx. 99%
Contains approx. 1% 2-isomer.
[111-03-5] $C_{21}H_{40}O_4$ FW 356.5

NONANOYL-N-METHYLGLUCAMIDE — 500 mg; 1 g; 5 g; 25 g
N 1138 (MEGA-9)
Approx. 98%
[85261-19-4] $C_{16}H_{33}NO_6$ FW 335.4

n-NONYL β-D-GLUCOPYRANOSIDE — 250 mg; 500 mg; 1 g; 5 g
N 7507 **Approx. 98%**
[69984-73-4] $C_{15}H_{30}O_6$ FW 306.4

OCTANOYL-N-METHYLGLUCAMIDE — 500 mg; 1 g; 5 g; 25 g
O 3129 (MEGA-8; OMEGA)
Approx. 98%
[85316-98-9] $C_{15}H_{31}NO_6$ FW 321.4

n-OCTYL α-D-GLUCOPYRANOSIDE — 100 mg; 500 mg; 1 g; 5 g; 10 g; 25 g; 100 g
O 0630 (n-Octyl α-glucoside)
Minimum purity 98%
Ref.: Brown, G.M., et al., Canadian J. Chem. **48**, 2525 (1970).
[29781-80-4] $C_{14}H_{28}O_6$ FW 292.4

n-OCTYL β-D-GLUCOPYRANOSIDE — 100 mg; 250 mg; 500 mg; 1 g; 5 g; 10 g; 25 g; 100 g
O 8001 (n-Octyl glucoside)
>98% (GC)
Ref: Gould, R.J., et al., Biochem., **20**, 6776 (1981).
[29836-26-8] $C_{14}H_{28}O_6$ FW 292.4

OCTYL β-D-THIOGLUCOPYRANOSIDE — 500 mg; 1 g; 5 g; 10 g
O 6004 **Crystalline**
Ref.: Saito, S. and Tsuchiya, T., Biochem. J., **222**, 829 (1984).
[85618-21-9] $C_{14}H_{28}O_5S$ FW 308.4

POLYOXYETHYLENE ETHERS

2 Cetyl Ether (Brij 52) (pfs) — 100 g; 500 g; 1 kg
P 3769 Contains antioxidants.
[9004-95-9]

10 Cetyl Ether (Brij 56) (pfs) — 100 g; 500 g; 1 kg
P 5759 Contains antioxidants.
[9004-95-9]

20 Cetyl Ether (Brij 58) (pfs) — 100 g; 500 g; 1 kg
P 5884 *[9004-95-9]*

For shipping restrictions and related costs see page 5.

(Continuation of)
POLYOXYETHYLENE ETHERS

		US $
	4 Lauryl Ether (Brij 30) (pfs) *[5274-68-0]*	100 g 8.40 500 g 19.50 1 kg 32.40 FOB Sigma
	9 Lauryl Ether (pfs) (Polidocanol) *[9002-92-0]*	10 g 8.25 50 g 25.65 100 g 42.05 FOB Sigma
	0 Lauryl Ether (pfs) *[9002-92-0]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	23 Lauryl Ether (Brij 35) (pfs) Suitable for use in Stein-Moore chromatography. Ref.: Stein, W.H. and Moore, S., J. Biol. Chem., 211, 893 (1954). *See also:* Brij 35 Solution, 30% w/v solution for use in various automated procedures *Page 181* *[9002-92-0]*	100 g 5.65 500 g 10.75 1 kg 18.90 5 kg 74.85 FOB Sigma
	2 Oleyl Ether (Brij 92) (pfs) Contains antioxidants. *[9004-98-2]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	0 Oleyl Ether (Brij 96) (pfs) *[9004-98-2]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	0 Oleyl Ether (Brij 99) (pfs) Contains antioxidants. *[9004-98-2]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	Stearyl Ether (Brij 72) (pfs) *[9005-00-9]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	Stearyl Ether (Brij 76) (pfs) Contains antioxidants. *[9005-00-9]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	Stearyl Ether (Brij 78) (pfs) *[9005-00-9]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	Stearyl Ether (Brij 721) (pfs) *[9005-00-9]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	Oleyl Ether (pfs) *[9004-98-2]*	100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma
	For use as a solubilizing for a variety of membrane and enzymes or in solutions that call for lubrol	10 g 4.50 100 g 8.25 500 g 19.30 1 kg 32.10 FOB Sigma

POLYOXYETHYLENESORBITAN

		US $
P 1379 BT	**Monolaurate (Tween 20)** (pfs) Syrup Fatty acid composition: Lauric acid approx. 55%; balance primarily myristic, palmitic, and stearic acids. *[9005-64-5]*	100 ml 8.05 500 ml 11.30 1 gal 36.20 FOB Sigma
P 1754 BT	**Monooleate (Tween 80)** (pfs) Syrup Fatty acid composition: Oleic acid approx. 75%; balance primarily linoleic, palmitic, and stearic acids. *See also:* Tissue Culture Media and Reagents *Page 1422* *[9005-65-6]*	100 ml 8.05 500 ml 11.30 1 gal 36.20 FOB Sigma
P 1504 BT	**Monopalmitate (Tween 40)** (pfs) Syrup Fatty acid composition: Palmitic acid approx. 90%; balance primarily stearic acid *[9005-66-7]*	100 ml 8.05 500 ml 11.30 1 gal 36.20 FOB Sigma
P 1629 BT	**Monostearate (Tween 60)** (pfs) Fatty acid composition: Stearic acid approx. 55%; balance primarily palmitic acid. *[9005-67-8]*	100 ml 8.05 500 ml 11.30 1 gal 36.20 FOB Sigma
P 4634 BT	**Trioleate (Tween 85)** (pfs) Fatty acid composition: Oleic acid approx. 70%; balance primarily elaidic, linolenic and palmitic acids. *[9005-70-3]*	100 ml 8.05 500 ml 11.30 1 gal 36.20 FOB Sigma

SORBITAN

		US $
S 6635 BT	**Monolaurate (Span 20)** (pfs) Fatty acid composition Lauric acid (C12:0) approx. 56%; balance primarily myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids. *[1338-39-2]*	250 ml 11.65 1 liter 37.10 FOB Sigma
S 6760 BT	**Monooleate (Span 80)** (pfs) Fatty acid composition: Oleic acid (C18:1) approx. 75%; balance primarily linoleic (C18:2), linolenic (C18:3) and palmitic (C16:0) acids. *[1338-43-8]*	250 ml 11.65 1 liter 37.10 FOB Sigma
S 6885 BT	**Monopalmitate (Span 40)** (pfs) Fatty acid composition: Palmitic acid (C16:0) approx. 90%; balance primarily stearic acid (C18:0). *[26266-57-9]*	250 g 11.65 1 kg 37.10 FOB Sigma
S 7010 BT	**Monostearate (Span 60)** (pfs) Fatty acid composition: Stearic acid (C18:0) approx. 50%; balance primarily palmitic acid (C16:0). *[1338-41-6]*	250 g 11.70 1 kg 37.30 FOB Sigma

(Continued)

For information on the use of this price list see page 7

PRODUCT NUMBER | US $

(Continuation of)
SORBITAN

S 3386 [BT] **Sesquioleate** (pfs) (Arlacel 83) — 250 ml 11.65; 1 liter 37.10 FOB Sigma
Fatty acid composition: Oleic acid (C18:1) approx. 70%; balance primarily palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2).
[8007-43-0]

S 7135 [BT] **Trioleate (Span 85)** (pfs) — 250 ml 11.65; 1 liter 37.10 FOB Sigma
Fatty acid composition: Oleic acid (C18:1) approx. 74%; linoleic acid (C18:2) approx. 7%; linolenic acid (C18:2) approx. 2%; palmitoleic acid (C16:1) approx. 7%; balance primarily palmitic acid (C16:0).
[26266-58-0]

TERGITOL (pfs) — 100 ml 5.40; 500 ml 9.70 FOB Sigma
[BT] Polyglycol Ether surfactants
Tergitol is a registered trademark of Union Carbide Chemicals and Plastics Co., Inc.
See also Biological Detergents, Anionic *Page 1540*

NP-7 **Type NP-7**
[127087-87-0]

NP-9 **Type NP-9**
[127087-87-0]

NP-10 **Type NP-10**
[127087-87-0]

NP-14 **Type NP-14**
[127087-87-0]

NP-35 **Type NP-35**
[127087-87-0]

NP-40 **Type NP-40**
[127087-87-0]

TMN-6 **Type TMN-6**
[60828-78-6]

TMN-10 **Type TMN-10** (Approx. 90% solution)
[60828-78-6]

XD **Type XD**
[9038-95-3]

XH **Type XH**
[9003-11-6]

15-S-3 **Type 15-S-3**
[68131-40-8]

15-S-5 **Type 15-S-5**
[68131-40-8]

15-S-7 **Type 15-S-7**
[68131-40-8]

15-S-9 **Type 15-S-9**
[68131-40-8]

15-S-12 **Type 15-S-12**
[68131-40-8]

15-S-15 **Type 15-S-15**
[68131-40-8]

15-S-20 **Type 15-S-20**
[68131-40-8]

15-S-30 **Type 15-S-30**
[68131-40-8]

15-S-40 **Type 15-S-40**
[68131-40-8]

T 1135 **MIN FOAM 1x**
[68551-14-4]

T 1260 **MIN FOAM 2x**
[68551-14-4]

PRODUCT NUMBER

TRITON (pfs)
[BT] Various Polyoxyethylene Ethers and other surface-active compounds.
The following liquid types are usually available.
Triton is a registered trademark of Union Carbide and Plastics Co., Inc.
[9036-19-5]

X-15	X-207	B-1956
X-35	X-301	CF-10
X-45	X-305	CF-21
X-102	X-705 (70%)	CF-32
X-151	XQS-20	CF-54
X-155	N-42	DF-12
X-165	N-57	DF-16
X-200	N-60	GR-5M

X-67 (solid flake) (pfs)
[59030-15-8]

All of the following package sizes [BT] each of the following products.

X-100 (pfs)
X-114
X-405
N-101
[9002-93-1]

X-100R-S
[BT] Triton X-100 hydrogenated to reduce UV absorption.
A_{275} (0.5% aqueous) ≤0.25
[101013-07-4]

X-405R (pfs)
Reduced
Not to be confused with Triton X-405.
[101013-07-4]

TYLOXAPOL (pfs)
T 8761 [BT] A nonionic liquid polymer of the alkyl aryl polyether alcohol type
[25301-02-4]

U 5254 [OC] **n-UNDECYL β-D-GLUCOPYRANOSIDE**
Approx. 97%
[70065-86-6] $C_{17}H_{34}O_6$, FW 33

For shipping restrictions and related costs see page 5.

PCT

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(54) Title: METHOD FOR PREPARING VIRUS–SAFE PHARMACEUTICAL COMPOSITIONS

(57) Abstract

The present invention concerns a method of preparing pharmaceutical compositions of a biologically active proteins, in particular multicomponent interferon compositions. The invention comprises the steps of adding to a solution of the protein a non–ionic detergent in an efficient amount to provide an extended shelf–life of the pharmaceutical composition; subjecting the solution containing the non–ionic detergent to filtration on a virus removal filter with a pore size of 10 to 40 nm; and recovering the filtrate. The method gives rise to, e.g., a virus–safe multicomponent α–interferon composition, comprising a non–ionic detergent as a stabilizer in an amount exceeding the critical micellar concentration of the detergent and being essentially free from substances retained on a virus–filter having high virus retentive capacity.

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METHOD FOR PREPARING VIRUS-SAFE PHARMACEUTICAL COMPOSITIONS

Background of the Invention

Field of the Invention

The present invention relates to the preparation of virus-safe pharmaceutical compositions of biologically active proteins. In particular, the present invention concerns a method for preparing a virus-safe, liquid formulation of α-interferon, preferably multicomponent α-interferon, having extended shelf-life. The present invention also relates to the use of non-ionic detergents as stabilizers of pharmaceutical compositions and to virus-safe multi-component α-interferon solutions which can be used as injectables in the treatment of diseases.

Description of Related Art

Pharmaceutical compositions of biologically active proteins must be virus-safe, i.e. they must be free from any contaminating, potentially pathogenic viruses and other infectious agents. Further, such pharmaceutical compositions should have extended shelf-life providing for their use over a prolonged period of time. In the following, the questions of virus-safety and shelf-life of proteineous pharmaceutical compositions will be discussed with particular reference to interferon formulations. However, the principles are generally applicable to physiologically active substance originating from human or animal blood, urine or internal organs and to corresponding recombinant proteins produced in cultured animal cells or transgenic animals.

Human alpha-interferons (IFN-α) comprise a family of closely related proteins with antiproliferative, antiviral and immunomodulatory effects. Human leukocytes and lymphoblastoid cells are known to produce several IFN-α subtypes in culture when induced by Sendai virus (Cantell et al., Methods Enzymol. 78, 29-38, 1981, Mizrani, Methods Enzymol. 78, 54-68, 1981). Purified multicomponent IFN-α drugs are used in the treatment of various diseases, including neoplastic and viral diseases. It has been shown in the art that multicomponent IFN-α drugs have therapeutic benefits in comparison with recombinant IFN-α drugs produced in bacteria, which only contain a single IFN-α subtype.

Commercial production of human multicomponent IFN-α comprises culturing human leukocytes or lymphoblastoid cells and inducing them with Sendai virus. These products therefore carry a risk of virus contamination. Blood-borne viruses potentially present in leukocytes and serum or its fractions used in the culture medium include HI-viruses, hepatitis C and B viruses and small non-enveloped viruses, such as parvovirus B19, which is resistant to many physicochemical treatments. Lymphoblastoid cell lines may harbour e.g. retroviruses. Production of IFN-α and other biologically active proteins in animal cell cultures or in transgenic animals also carries a risk of viral contamination.

An effective method for the removal of viruses of diverse physicochemical properties is filtration with membranes with high virus retentive capacities, also known as nanofiltration or virus filtration. The particular advantage of filtration is that it will also remove viruses, such as non-enveloped viruses, and other infectious agents, such as those causing transmissible spongiform encephalopathies ("prions"), which exhibit resistance to conventional treatments based on the use of heat and chemicals (physicochemically resistant agents).

In order to prevent the binding of biologically active proteins, such as IFN-α, to filters, final containers and other surfaces, stabilizers are typically added to solutions containing the purified biologically active protein. In addition to the above short-term stabilizing effect, stabilizers will also prevent aggregation of the proteins and, thus, provide extended shelf-life. Albumin is the most common stabilizer used, e.g., in multicomponent IFN-α products and it is employed in many of the commercial preparations (Alfanative®, Alferon® N, Wellferon®).

However, the use of albumin as a stabilizer in IFN-α products may cause at least two problems. First, albumin has been reported to result in the formation of albumin-IFN aggregates in the product, which may be antigenic and result in the formation of antibodies against IFN-α (Braun et al., Pharm. Res. 14, 1472-1478, 1997). These problems have been identified with bacterial recombinant IFN-α products. Second, and importantly as regards the preparation of virus-safe formulations, if the formulated IFN-α solution is to be filtrated with a virus removal filter, as is the case for IFN-α compositions produced in human or animal cells or in transgenic animals, the use of albumin as a stabilizer decreases the ability of the filter to remove viruses, since it has been shown that virus removability of a virus removal filter decreases with increasing concentration of coexisting protein (Hirasaki et al., Membrane 20, 135-142, 1995). This is evidently caused by plugging of the

filter with coexisting protein which is reflected as decreasing filtration rate when pressure is kept constant. As Example 2 below shows, the filtration rate dropped by about 80 % after filtration of 20 l/m^2 of a highly purified IFN-α solution containing 1 g/l albumin.

It is known in the art that certain proteins, in particular human growth hormone, can be prevented from adsorbing onto a membrane filter by pretreating the filter with human serum albumin or with polyvinylpyrrolidone, polyoxyethylene sorbitan monolaurate, polysorbate 80, modified gelatin and gelatin (US Patent No. 5,173,415). This known pretreatment comprises adsorbing albumin or another of the listed substances to the filter from an aqueous solution by filtration, impregnation or soaking.

Although said treatment may have some beneficial effect on the filtration rate, it constitutes an additional, cost-consuming step. Furthermore, the coating of the filter with albumin will not reduce adsorption of the proteins to other surfaces being in contact with the product, such as tubing, collecting vessels, vials and stoppers.

Summary of the Invention

It is an object of the present invention to eliminate the problems of the prior art and to provide a novel method of preparing virus-safe pharmaceutical compositions of biologically active proteins.

It is another object of the invention is to provide a new use of non-ionic detergents as stabilizers for liquid formulations of biologically active proteins, such as IFN-α, which can be filtered with a virus removal filter with improved yield and capacity and used as injectables.

It is a third object of the present invention to provide a novel liquid formulation of multi-component IFN-α, which does not contain polymers of IFN-α or albumin-IFN complexes, which exhibits prolonged shelf-life and which can be used as an injectable.

These and other objects, together with the advantages thereof over known processes, which shall become apparent from specification which follows, are accomplished by the invention as hereinafter described and claimed.

The present invention is based on the finding that by using a non-ionic detergent as a

stabilizer of pharmaceutical compositions comprising biologically active proteins and by adding said stabilizer to the formulation before virus filtration, the yield and capacity of virus filtration can be greatly increased. This finding was surprising since it is known that non-ionic surfactants, like polysorbate 80, have very low critical micelle concentrations (CMC). Thus, the CMC of for example polysorbate 80 is ca. 0.013 g/l in aqueous solutions (Helenius and Simons, Biochim. Biophys. Acta 415, 29-79, 1975). Above the CMC, non-ionic surfactants form micelles with varying sizes, which penetrate very slowly e.g. dialysis membranes.

According to the present invention, non-ionic detergents (surfactants) are added to pharmaceutical compositions in concentrations above the CMC before virus removal filtration to provide stabilized proteineous formulations, for example multicomponent IFN-α formulations, which are essentially free from substances (including viruses and prions) having a size in excess of 10 to 40 nm, in particular 10 to 20 nm, and normally being retained on a virus filter.

In particular, the present method for preparing virus-safe pharmaceutical compositions of biologically active proteins is characterized by what is stated in the characterizing part of claim 1.

The method for stabilizing pharmaceutical compositions of purified leukocyte α- interferon is characterized by what is stated in the characterizing part of claim 13 and the virus-safe α-interferon solution is characterized by what is stated in the characterizing part of claim 15.

The invention provides considerable advantages. Thus, a multicomponent IFN-α solution stabilized according to the present invention with a non-ionic detergent exhibits improved stability. Further, multicomponent IFN-α formulations stabilized with a non-ionic detergent do not contain albumin-IFN complexes, which are formed in albumin-containing formulations and are suggested to be harmful in recombinant IFN-α products. By replacing albumin with a non-ionic detergent as a stabilizer, an IFN-α solution can be filtered with a virus removal filter without plugging of the filter. In other words, by substituting a non-ionic detergent for albumin, it is possible to filter IFN-α solution with improved yield and capacity with a virus removal filter. In comparison to the method known from US Patent No. 5,173,415, the present invention not only increases the yield of filtration, it also prevents losses caused by adsorption of protein from the filtrate to other surfaces being in

contact with the product, such as tubing, collecting vessels, vials and stoppers. By incorporating the non-ionic detergent in the composition before filtering, no pretreatment of the filter is necessary. In fact, test have shown, that such a pretreatment will not improve the yield to any discernible extent.

Next, the invention will be examined more closely with the aid of the following detailed description and with reference to a number of working examples.

Brief Description of the Drawings

In the attached drawings,
Figure 1 shows the adsorption of IFN-α to glass in the presence of different stabilizers;
Figure 2 shows the occurrence of albumin-IFN complexes in solutions stabilized with albumin and the lack of aggregates in solutions stabilized with polysorbate 80;
Figure 3 depicts the virus filtration flow rates of purified IFN-α solutions stabilized by polysorbate 80 and albumin, respectively.

Detailed Description of the Invention

According to the present invention, a non-ionic detergent is added to a solution of purified biologically active protein, which is subsequently filtered with a virus removal filter having a pore size of about 10 to 40 nm and then optionally sterile filtered to obtain a virus-safe, sterile and stable protein solution.

The scope of biologically active proteins covered by the present invention extends to all therapeutically used proteins which may harbour viruses and which are filtered with a virus removal filter. Such proteins generally have a molecular weight of less than 180,000 D and include coagulation factors and their activated forms (e.g. factor IX, factor VII), proteinases, their activated forms and proteinase inhibitors (e.g. protein C), growth factors and colony stimulating factors (e.g. IGF-1, G-CSF, GM-CSF), neurotrophic factors (e.g. NGF, GDNF, NT-3), hormones (e.g. erythropoietin, growth hormone) and other proteins modifying the biological response of cells (e.g. interferons and interleukins). Not only naturally occurring proteins should be considered but also recombinant proteins produced in cultured animal cells or transgenic animals.

The use of non-ionic detergents in various pharmaceutical compositions is known *per se*. It

has also been suggested in the art to use polysorbate 80 instead of albumin as a stabilizer of a recombinant IFN-α2a product in order to prevent formation of albumin-IFN aggregates (Hochuli, J. Interferon Cytocine Res. 17, Suppl. 1, S15-S21, 1997). Liquid α- and γ-interferon compositions containing non-ionic detergents are also disclosed in EP Patent Application No. 0 736 303 A2 and WO 89/04177. However, all the citations are completely silent about the incorporation of a non-ionic detergent into a pharmaceutical composition prior to virus-filtration.

According to a preferred embodiment of the present invention, non-ionic detergents are used as stabilizers of multicomponent IFN-α formulations subjected to virus filtration for removing any agents retained on filters having a pore size of 10-40 nm. These compositions comprise purified leukocyte and lymphoblastoid interferons containing two or more of the following IFN-α subtypes: α1, α2, α4, α7, α8, α10, α14, α17 and α21. Human leukocyte interferon has been shown to contain at least nine IFN-α subtypes (Nyman et al., Biochem. J. 329, 295-302, 1998), and lymphoblastoid interferon contains the same or similar subtypes (Zoon et al., J. Biol. Chem. 267, 15210-15216, 1992). Part of the subtypes secreted by the producer cells may be lost during purification, depending on the purification process employed (US Patent 5,503,828).

Methods for the production of multicomponent IFN-α have been described in detail before. Multicomponent IFN-α can be produced in leukocyte or lymphoblastoid cell cultures by Sendai virus induction. IFN-α subtypes with close structural similarity to the natural subtypes can be produced by recombinant DNA technology in cultured animal cells or in transgenic animals. The process for manufacturing a highly purified drug substance may consist of precipitations, filtrations and chromatographic steps. Purification methods of multicomponent IFN-α employing monoclonal or polyclonal antibodies have also been disclosed. The manufacturing process may contain additional virus inactivation steps, such as treatment with low pH and solvent/detergent treatment. IFN-α composition and methods for its production from human peripheral blood leukocytes are disclosed in, e.g. US Patents Nos. 5,503,828 and 5,391,713, the contents of which are herewith incorporated by reference.

A purification process yielding all major IFN-α subtypes is described in Example 2. Generally, it comprises, e.g., the step of contacting a solvent/detergent treated composition with at least two monoclonal mouse IgG antibodies having complementary subtype specificities in an immunoadsorption step. The α-interferon subtypes bound by the

monoclonal antibodies are eluted and the eluate is purified and filtered on a virus removal filter.

Other pharmaceutically useful proteins which can be subjected to virus removal filtration can be produced by methods known *per se*, for example by isolating from human or animal blood or by recombinant DNA technology in cultured cells or transgenic animals.

According to the present invention, a formulated protein solution is prepared by diluting a calculated amount of the purified biologically active protein with a formulation buffer containing polysorbate 80 or another non-ionic detergent in an amount, which gives a final concentration of 0.05 to 1 g/l, preferably about 0.1-0.5 g/l, of the non-ionic detergent. The degree of purity of the protein is advantageously at least about 90 %. The formulated solution may be prefiltered with a 0.04-0.2 μm filter and thereafter filtered with a virus removal filter having a preferred pore size of 10-40 nm. The non-ionic detergent does not cause any plugging of the filter and, depending on the molecular size of the protein, the filtration can be carried out with a constant pressure without any decrease in the filtrate flux and thus with high capacity and constant removability of viruses. Two virus filters may be used sequentially, which improves virus removal.

The recovered filtrate is filtered with a sterile filter and filled in vials, syringes or other containers compatible with parenteral injectables. It is also possible to carry out the virus filtration and sterile filtration in reversed order.

Included in the scope of a virus removal filter (nanofilter) are filters suitable for the removal of viruses from pharmaceutical proteins solutions. The size of the pores or perforations in the filter should be small enough to effectively remove even small non-enveloped viruses, such as parvoviruses. The proper pore size can be assessed by spiking experiments with model viruses, in which at least 4 log, preferably at least 6 log, of model viruses with a size of ca 20 to 40 nm should be removed. Based on such tests, the theoretical pore sizes of the virus removal filters can be estimated to be about 10 to 40 nm, preferably about 10 to 20 nm. In the present context, virus filters capable of reducing the concentration of model viruses at the above mentioned spiking tests with at least 4 log, are considered to have a "high virus retentive capacity". It is particularly important that the filters used have such capacity also in relation to small non-enveloped viruses.

The buffer of the liquid formulation is less critical and may be an inorganic buffer or

organic buffer. The pH of the buffer may be in the range of 4.5-7.5, and the buffer may contain other substances, e.g. inorganic salts, sugars, amino acids, polyols or cyclodextrins. Other stabilizers can be added to IFN-α solution after the virus filtration step.

The activity of IFN-α solution to be filtered with a virus removal filter may be close to that in the final product or it may be considerably higher. In the latter case, the solution is diluted after virus filtration. The activity of IFN-α in the final product is selected based on several variables, including the disease to be treated, therapeutic regimen and administration system. Generally, the activity of IFN-α solution before virus filtration is in the range of 3 to 50 mill. IU/ml.

Examples of non-ionic detergents to be used as a stabilizer include polyoxyethylene-based detergents, such as polyoxyethylene sorbitan monooleate (polysorbate 80), polyoxyethylene sorbitan monolaurate (polysorbate 20), polyoxyethylene lauryl ethyl (laureth 4) and polyoxyethylene, polyoxypropylene block polymer (poloxamer 188). Polysorbate, such as polysorbate 80 is most preferred. Polysorbate 80 as well as the other non-ionic detergents are used at concentrations in excess of the critical micellar concentration, in the case of polysorbate 80 typically about 0.05 to 1 g/l. A preferred range is 0.1-0.5 g/l, and most preferred concentration about 0.2 g/l.

According to a preferred embodiment the non-ionic detergent used has a low peroxide number, so as to prevent any harmful oxidation reactions in the pharmaceutical formulations. Preferably, the peroxide number is less than 5.0 mEq/kg tested according to Ph. Eur. 1997. Optionally, an antioxidant can be added to the formulation in order to prevent oxidation of IFN-α.

The following non-limiting Examples illustrate the invention:

Analytical Methods used in the Examples

IFN-α concentration

The IFN-α concentration was measured by a time-resolved fluoroimmunoassay (FIA) on microtitre plates. The IgG fraction of a bovine antiserum against human leukocyte IFN-α was used in capturing and a mixture of two Eu-labelled mouse IgG monoclonal antibodies to IFN-α for detection. The monoclonal antibodies were the same as used in the purification of IFN-α (Example 1). The details of the assay have been described elsewhere

(Rönnblom et al., APMIS 105, 531-536, 1997). IFN-α concentration was expressed as IU/ml using a laboratory standard, which was calibrated by the virus plaque reduction assay against the International Reference Preparation of Interferon, Human Leukocyte 69/19 (NIBCS, U.K.).

Interferon antiviral activity

The antiviral activity of the IFN was determined by a virus plaque reduction assay in 35 mm petri dishes using Human Epithelial 2 (HEp2) cells challenged with Vesicular stomatitis virus (VSV). The IFN-α samples, control and standard were diluted serially at 0.25 log intervals to concentration of 0.3-3 IU/ml in Eagle's Minimum Essential Medium (EMEM) supplemented with fetal calf serum (FCS) 7% and aureomycin 0.004%. The samples were assayed as triplicates at four dilutions in at least two assay series. One ml of cell suspension (2 x 10^6 cells/ml) in EMEM and 1 ml of sample dilution were added to dishes. Virus control dishes without IFN were included in each assay series. After incubation of overnight at 37 °C in 3-4% CO_2 atmosphere the solutions were removed from the confluent cell layers and 150-200 PFU of VSV in 1 ml of EMEM was added. After incubation of 40-45 min the virus was removed and cells were overlayed with 2 ml of agar 0.8% in EMEM. After overnight incubation the virus plaques were calculated. One unit of IFN activity is the highest dilution of the sample, which inhibits 50% of the virus plaques as compared to the virus control. Interferon activity was expressed in International Units (IU) using a laboratory standard, which was calibrated against the International Reference Preparation of Interferon, Human Leukocyte 69/19 (NIBCS, UK).

Total protein

Total protein concentration was measured according to Lowry using human albumin as a standard (Total Protein Standard, Finnish Red Cross Blood Transfusion Service, Helsinki, Finland).

Western blot

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli using 15% gels. Proteins were electroblotted to a nitrocellulose membrane, the membrane was blocked with 0.5% Tween 20 and washed with 0.05% Tween 20 in 0.011 mol/l sodium phosphate buffer, pH 7.0, containing 0.14 mol/l NaCl (PBS). The membrane was incubated with bovine polyclonal IgG against IFN-α (Wellcome Research Laboratories), 4 µg/ml in PBS containing 0.05% Tween 20 and 0.1% human albumin for 2 h at room temperature. The membrane was washed and incubated

with peroxidase-conjugated rabbit anti-bovine IgG (Jackson Immunoresearch Laboratories, PA, USA). After washing, the positive bands were visualized by using 4-chloro-1-naphthol as the peroxidase substrate.

Polysorbate 80

Polysorbate 80 concentration was measured by a colorimetric method (Milwidsky, Analyst 94, 377-386, 1969).

Example 1

Production of purified leukocyte IFN-α

This example describes the production of a high purity leukocyte IFN-α drug substance which was used in the stabilization and filtration examples (Examples 2-5).

The production of crude interferon was carried out in leukocyte cultures induced by Sendai virus essentially as described before (Cantell et al., Methods Enzymol. 78, 29-38, 1981). Residual cells in the culture supernatant were removed by microfiltration and the filtrate was concentrated 20-fold by ultrafiltration. The crude IFN concentrate was filtered through 1.2 μm and 0.22 μm filters and treated with 0.3% tri(n-butyl)phosphate and 1% polysorbate 80 for 16 h at 26 °C (solvent/detergent treatment). The solution was applied to an immunoadsorbent column containing two monoclonal antibodies against IFN-α coupled to CNBr-Sepharose 4FF gel. The monoclonal antibodies have complementary binding specificities and together bind all major IFN-α subtypes. The immunoadsorbent column was washed extensively and the bound IFN-α was eluted with buffer adjusted to pH 2. The eluate was neutralized and concentrated about 30-fold by ultrafiltration. The concentrated eluate was applied to a Superdex 75 gel filtration column equlibrated and eluted with PBS. The IFN-α containing fractions were pooled and the purified drug substance thus obtained was stored frozen at -70 °C.

The purified drug substance was analyzed for IFN-α subtype composition by using procedures described in detail elsewhere (Nyman et al., Biochem. J. 329, 295-302, 1998). It was found to contain the subtypes α1, α2, α4, α7, α8, α10, α14, α17 and α21.

Example 2

Short term adsorption of purified multicomponent IFN-α onto glass from different formulations

Short-term stabilizing effect of various stabilizers was determined by assessing the adsorption of IFN-α onto glass.

Purified leukocyte IFN-α bulk drug was diluted in polypropylene vials to a final concentration of 3 mill. IU/ml (0.02 g/l) in PBS containing one of the following stablizers:

1. polyoxyethylene lauryl ether (laureth 4, Brij® 35, CAS-9002-92-0)
2. polyoxyethylene sorbitan monooleate (polysorbate 80, Tween® 80, CAS-9005-65-6)
3. polyoxyethylene, polyoxypropylene block polymer (poloxamer 188, Pluronic® F-68, CAS-9003-11-6)
4. human serum albumin

Laureth 4, polysorbate 80 and poloxamer 188 were used at final concentrations of 0.1, 0.2, 0.5 and 1.0 g/l. Albumin was added to a final concentration of 0.5, 1.0, 1.5, and 2.0 g/l. As a control, the IFN-α bulk drug was diluted in PBS. Samples were taken from the formulated solutions immediately after mixing for the determination of IFN-α concentration, and 100 µl of the formulated solutions were transferred into glass vials. The vials were kept for 20 h at at room temperature (23 °C). Samples were taken for IFN-α concentration determination. The results are shown in Figure 1. Adsorption was determined as the difference between the initial and final concentration of IFN-α in the vials.

About 30 % of IFN-α was adsorbed onto the glass vials in the absence of any stabilizer (Fig. 1). The stabilizers studied prevented the adsorption of IFN-α to a different extent. Polysorbate 80 was most effective followed by laureth 4, albumin and poloxamer 188.

Formation of IFN-containing aggregates was studied by Western blot analysis under non-reducing conditions. Highly purified leukocyte IFN-α was incubated in glass vials in PBS containing polysorbate 80 or albumin for 20 h at at 23 °C. Figure 2 shows the Western blot of the samples containing 0.1 g/l (lane 3), 0.2 g/l (lane 4), and 0.5 g/l (lane 5) of polysorbate 80, and 0.5 g/l (lane 6), 1.0 g/l (lane 7) and 1.5 g/l (lane 8) of albumin. Lanes 1 and 2 show negative and positive IFN-α aggregate controls, respectively. In polysorbate

80-containing solutions only bands corresponding to IFN-α monomers and dimer were seen. The intensity of the dimer band was weaker at polysorbate 80 concentrations 0.2 g/l and 0.5 g/l than at 0.1 g/l. In albumin solutions dimer bands were more intensive and additionally, bands with higher molecular weight corresponding to albumin-IFN complexes were seen. In polysorbate 80 formulations no bands corresponding to higher molecular weight complexes could be detected.

Example 3

Comparison of polysorbate 80 and albumin in the manufacture of virus-filtered and sterile-filtered multicomponent IFN-α solutions

Purified leukocyte IFN-α was diluted to the activity of 5 mill. IU/ml (40 μg/ml) in PBS containing either 0.2 g/l polysorbate 80 or 1 g/l albumin. The formulated solutions were prefiltered with a 0.1 μm filter and subjected to virus filtration by using Planova 15N filters (Asahi Chemical Industry Co, Japan). Filtrations were carried out in tangential flow mode at room temperature with a constant pressure of 0.8 bar. The system was pressurized with nitrogen gas. At the end of the filtration the virus filter was washed with formulation solution in the dead-end mode in order to recover all product from the filter system. Pressure, temperature and the mass of the filtrate were recorded during filtration. Samples were taken from the formulated solutions, after prefiltration, after virus filtration, and after sterile filtration for the determination of IFN-α concentration, polysorbate 80 and total protein and Western blot assay.

The results are summarized in Table 1 below and in Figure 3. Table 1 indicates the yield of IFN-α in the manufacture of a virus-filtered finished product by using polysorbate 80 (0.2 g/l) or albumin (1 g/l) as a stabilizer.

Table 1. Yield of IFN-α in the manufacture of a virus-filtered finished product calculated from IFN-α FIA results

Manufacturing step	Cumulative yield of IFN-α (%)	
	Polysorbate solution (n=3)	Albumin solution (n=3)
Formulated IFN-α bulk solution	100	100
Prefiltrated solution	99	97
Planova 15 filtrated solution	102	88
Sterile filtrated solution	101	89

As apparent from Table 1, the yield of IFN-α in the virus-filtered and sterile-filtered solution was consistently better in the presence of 0.2 g/l polysorbate than in the presence of 1 g/l albumin. Most of the IFN-α loss in albumin solutions took place during virus filtration, whereas there was no significant loss of IFN-α in polysorbate solution at the corresponding step. Notably, the recovery of polysorbate 80 was 99 % in filtrate of the virus filtration, indicating that there was no retention tendency of polysorbate during virus filtration. The recovery of albumin in the filtrate was 87% indicating that albumin was retented by the filter.

Figure 3 depicts Planova 15N filtration flow rates of purified IFN-α solutions stabilized by polysorbate 80 or albumin. Purified leukocyte IFN-α (40 μg/ml) in PBS containing 0.2 g/l polysorbate 80 (open circles) or 1.0 g/l albumin (closed circles) was filtered with Planova 15N filter at a constant pressure of 0.8 bar in tangential flow mode. The filtration rate remained constant in the presence of polysorbate 80 at least during filtration of 200 l/m^2, whereas it was reduced by about 80% in the presence of 1 g/l albumin already after filtration of 20 l/m^2. This indicates that the filter became plugged when albumin-containing solution was filtered, whereas there was no plugging tendency when polysorbate-containing solutions were filtered. The same results were confirmed by filtering pure albumin and polysorbate solutions (data not shown). Filtration of polysorbate-containing solution could be performed also in dead-end mode without any decrease in filtrate flow. Virus filtration did not cause any changes in the molecular weight distribution of IFN-α as analyzed by Western blot.

Example 4

Manufacture of a virus-filtered IFN-α finished drug stabilized with polysorbate 80

A formulated IFN-α bulk solution was prepared by adding to a suitable container PBS and polysorbate 80, mixing them, and adding purified multicomponent IFN-α so that the desired IFN-α activity was obtained in the calculated final volume of PBS containing 0.2 g/l of polysorbate 80. The formulated IFN-α solution was mixed carefully and prefiltered with a 0.1 μm filter. The prefiltered IFN-α solution was filtered through a virus filter (Planova 15N, Asahi) at a constant pressure of 0.9 bar in a dead-end mode. The filtrate was recovered and filtered with a 0.1 or 0.22 μm sterile filter and filled aseptically into the final containers.

Example 5

Stability of the virus-filtered IFN-α solution containing polysorbate 80

The stability of the virus-filtered IFN-α finished product manufactured according to Example 4 was studied at 6 °C and at 25°C up to six months. The results are given in Table 2.

Table 2. Stability of virus-filtered IFN-α solution stabilized with 0.2 g/l polysorbate 80

Time point (months)	IFN-α concentration mean ± SD (mill. IU/ml)		IFN antiviral activity mean ± SD (mill. IU/ml)	
	6°C	25 °C	6 °C	25 °C
0	4.5 ± 0.1	4.5 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
1.5	4.6 ± 0.2	4.0 ± 0.1	4.5 ± 1.0	3.8 ± 0.0
3	4.5 ± 0.1	3.2 ± 0.0	4.3 ± 1.3	2.9 ± 0.5
6	4.3 ± 0.1	2.0 ± 0.0	4.4 ± 0.0	1.8 ± 0.2

As apparent from Table 2, no reduction in the immunochemical concentration and biological activity of IFN-α takes place during six months at 6 °C . A slight decrease (5-10 %) takes place at room temperature after storage for 1.5 months, and a decrease of about 30% is observed at room temperature after storage for 3 months. The results suggest good long-term stability for polysorbate-stabilized IFN-α solution stored at 2 to 8 °C.

Claims:

1. Method of preparing a virus-safe pharmaceutical composition of a biologically active protein, comprising the steps of

- adding to a solution of the protein a non-ionic detergent in an efficient amount to provide an extended shelf-life of the pharmaceutical composition;
- subjecting the solution containing the non-ionic detergent to filtration on a virus removal filter with a pore size of 10 to 40 nm; and
- recovering the filtrate.

2. The method according to claim 1, wherein the non-ionic detergent is selected from the group consisting of polyoxyethylene sorbitan mono-oleate, polyoxyethylene sorbitan monolaurate and polyoxyethylene lauryl ether.

3. The method according to claim 2, wherein the non-ionic detergent comprises polyoxyethylene sorbitan mono-oleate (polysorbate 80), which is added in an amount exceeding the critical micellar concentration.

4. The method according to claim 3, wherein polysorbate is added in an amount of 0.05 to 1 g/l.

5. The method according to any of claims 1 to 4, wherein the pharmaceutical composition comprises the solution of at least one protein selected from the group of coagulation factors, proteinases and proteinase inhibitors, growth factors and colony stimulating factors, neurotrophic factors, hormones and other proteins modifying the biological response of cells.

6. The method according to claim 5, wherein the pharmaceutical composition comprises the solution of purified α-interferon.

7. The method according to any of claims 1 to 6, wherein the activity of the α-interferon solution before virus filtration is in the range of 3 to 50 mill. IU/ml.

8. The method according to claim 6 or 7, wherein the pharmaceutical composition comprises an α-interferon solution containing at least one α-interferon subtype selected from the group consisting of α1, α2, α4, α7, α8, α10, α14, α17 and α21.

9. The method according to any of the preceding claims, comprising preparing a pharmaceutical composition comprising purified leukocyte or lymphoblastoid α-interferon essentially in the absence of α-interferon polymers and albumin-interferon complexes.

10. The method according to any of the preceding claims, comprising prefiltering a proteineous solution with a 0.04-0.2 μm filter, then filtering it with a virus removal filter having a pore size of 10-40 nm, and finally subjecting the filtrate to sterile filtration, and recovering the filtrate.

11. The method according to any of claims 1 to 9, comprising sterile filtering a proteineous solution and subsequently subjecting the filtrate of the sterile filtration to virus removal filtration with a filter having a pore size of 10 to 40 nm, and recovering the filtrate.

12. The method according to any of claims 1 to 11, comprising using a virus removal filter capable of reducing the concentration of model viruses having a size of ca 20 to ca 40 nm with at least 4 log during a spiking test.

13. Method of stabilizing pharmaceutical compositions of purified leukocyte α-interferon subjected to filtration on a virus removal filter, comprising using a polysorbate as a stabilizer.

14. A virus-safe α-interferon composition, comprising a non-ionic detergent as a stabilizer in an amount exceeding the critical micellar concentration of the detergent and being essentially free from substances and agents retained on a virus-filter having a high virus retentive capacity even for small non-enveloped viruses.

15. The composition according to claim 14, comprising an α-interferon solution containing at least one α-interferon subtype selected from the group consisting of α1, α2, α4, α7, α8, α10, α14, α17 and α21, and containing a polysorbate as a stabilizer in an amount of 0.05 to 1 g/l.

16. The composition according to claim, comprising an α-interferon solution containing at least two α-interferon subtypes selected from the group consisting of α1, α2, α4, α7, α8, α10, α14, α17 and α21.

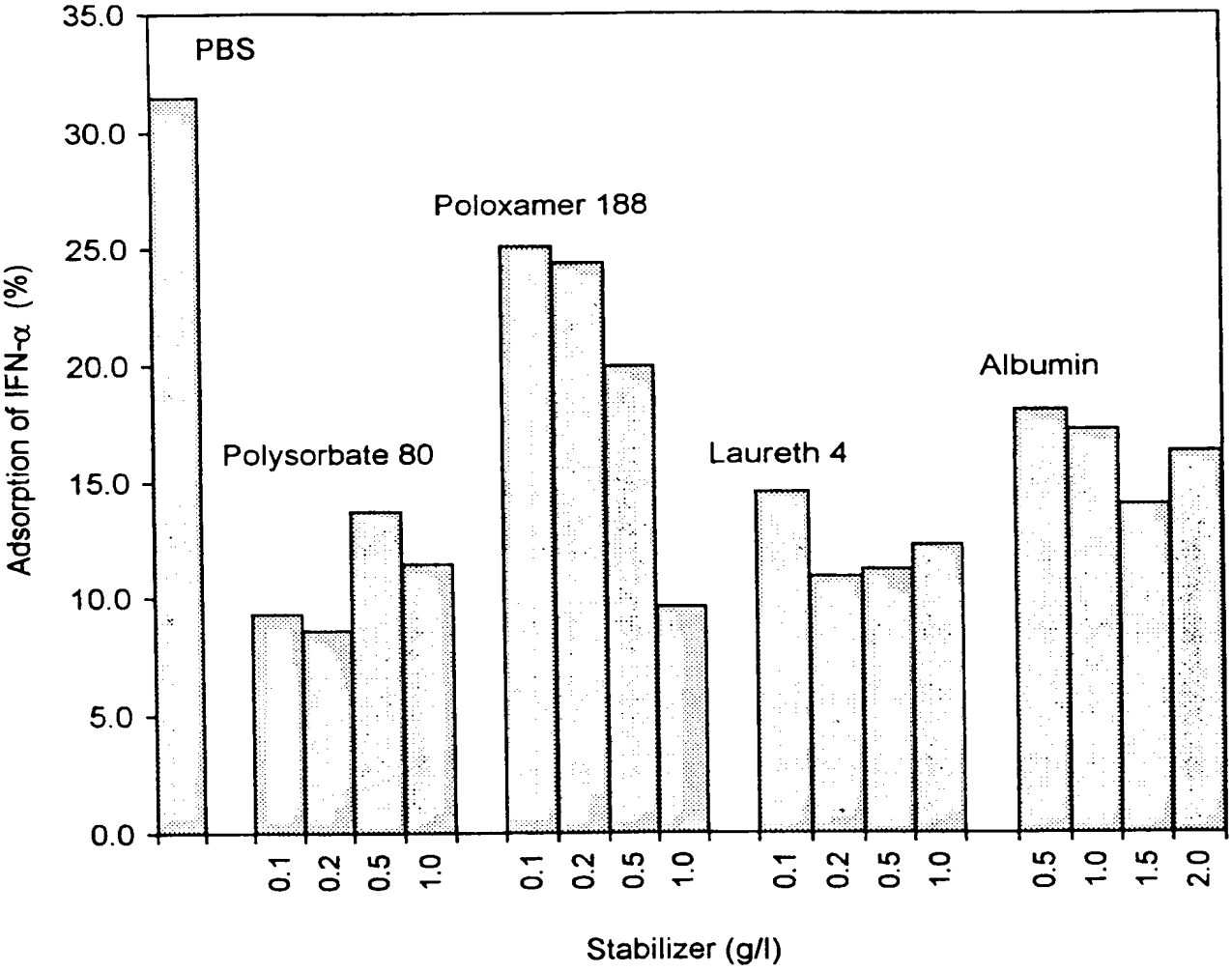
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Adsorption of IFN-α (%)
PBS
Polysorbate 80
Poloxamer 188
Laureth 4
Albumin
0.1
0.2
0.5
1.0
0.1
0.2
0.5
1.0
0.1
0.2
0.5
1.0
0.5
1.0
1.5
2.0
Stabilizer (g/l)

FIG. 1

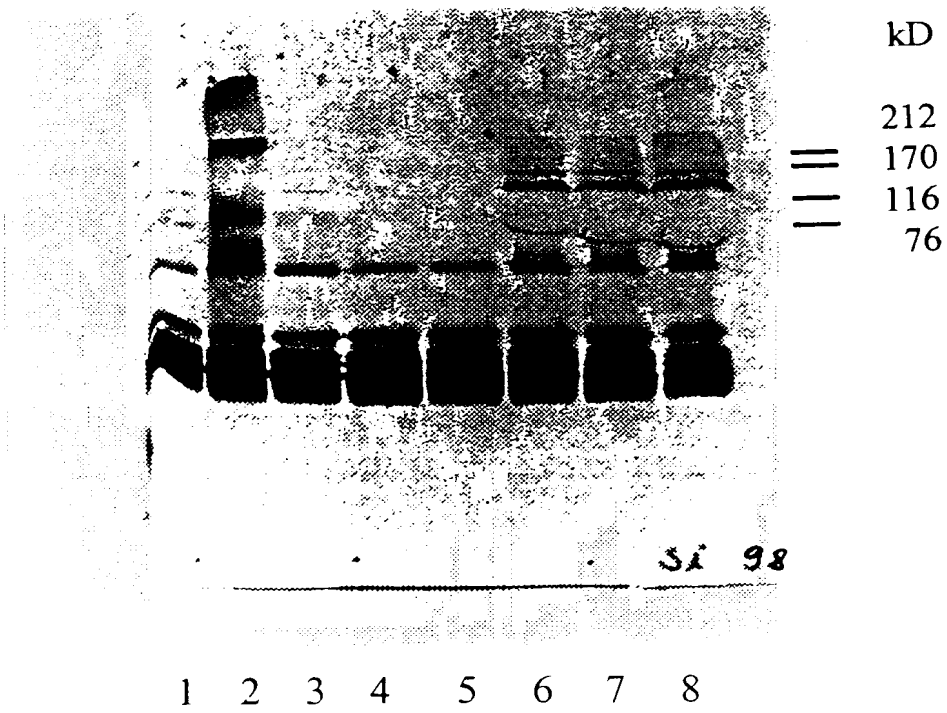

FIG. 2

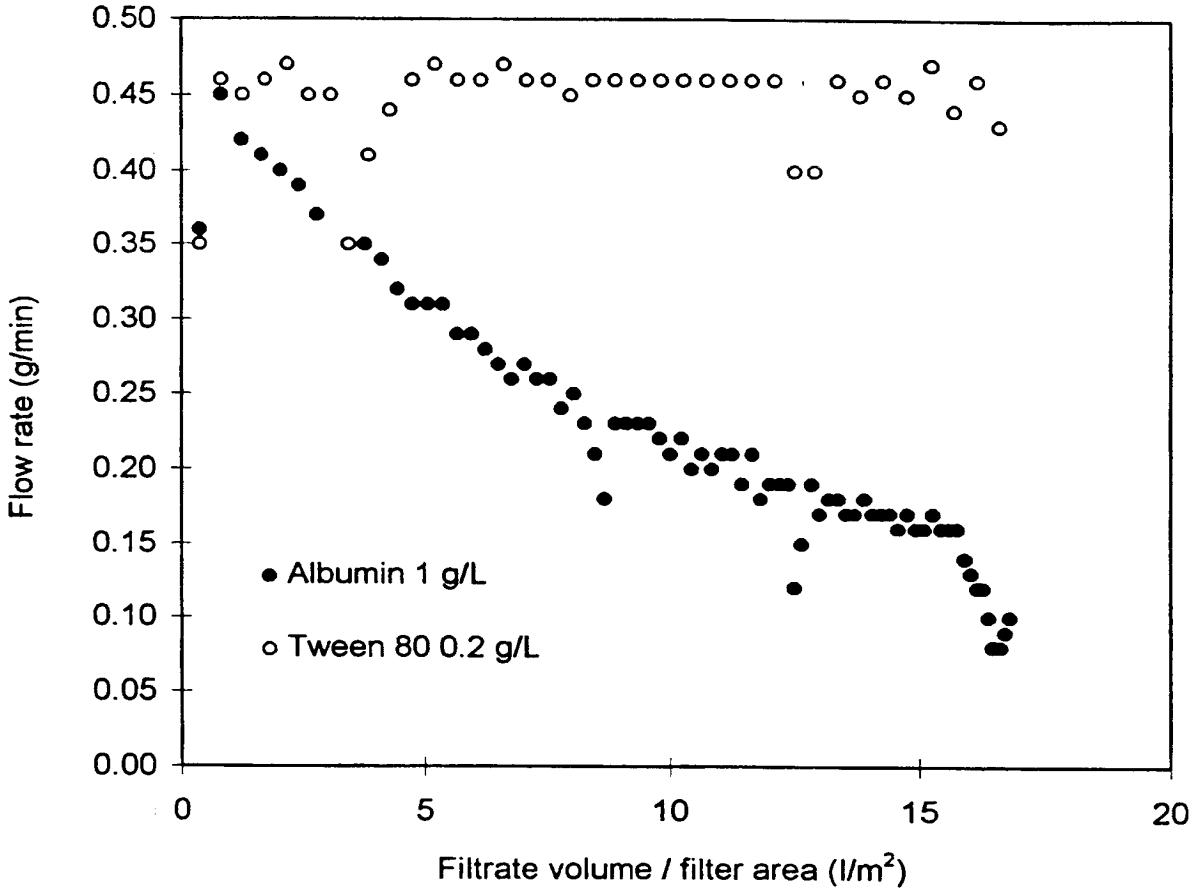
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Flow rate (g/min)
Albumin 1 g/L
Tween 80 0.2 g/L
0
5
10
15
20
Filtrate volume / filter area (l/m²)

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00505

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/34, C07K 14/56, A61K 38/21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0152345 A2 (INTERFERON SCIENCES, INC A DELAWARE CORPORATION), 21 August 1985 (21.08.85), see abstract; page 17, line 25 - page 18, line 1; example 2 --	1-16
X	EP 0231816 A2 (DR. KARL THOMAS GMBH), 12 August 1987 (12.08.87), see abstract; example 5 --	1-16
A	EP 0571871 A2 (SEITZ-FILTER-WERKE GMBH UND CO.), 1 December 1993 (01.12.93), see abstract; page 3, lines 10-14; page 6, lines 16-20 -- --------	1-16

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 Sept 1999	15 -09- 1999
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/EÖ Telephone No. + 46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/08/99

International application No.
PCT/FI 99/00505

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0152345 A2	21/08/85	SE 0152345 T3	
		AT 91901 T	15/08/93
		CA 1254832 A	30/05/89
		DE 3587479 A,T	02/09/93
		JP 1978803 C	17/10/95
		JP 7005479 B	25/01/95
		JP 60188328 A	25/09/85
		US 4680175 A	14/07/87
		US 4911908 A	27/03/90
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		AT 63823 T	15/06/91
		AU 601712 B	20/09/90
		AU 6829287 A	06/08/87
		CA 1295242 A	04/02/92
		DD 284602 A	21/11/90
		DE 3603444 A	06/08/87
		DK 58387 A	06/08/87
		DK 164202 B,C	25/05/92
		FI 86144 B,C	15/04/92
		FI 870457 A	06/08/87
		GR 3002270 T	30/12/92
		IE 59697 B	23/03/94
		JP 62209024 A	14/09/87
		PH 24377 A	13/06/90
		PT 84243 A,B	01/03/87
EP 0571871 A2	01/12/93	DE 4217335 A,C	02/12/93
		DE 59304079 D	00/00/00
		ES 2092725 T	01/12/96
		US 5376274 A	27/12/94